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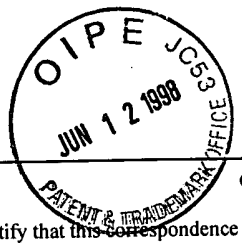

PATENT
Docket No. 212302000320

CERTIFICATE OF MAILING BY "FIRST CLASS MAIL"

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on June 9, 1998.

Valerie L. Cohen

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

S. Miltenyi et al.

Serial No.: 08/416,920

Filing Date: April 21, 1995

For: DIRECT SELECTION OF CELLS BY SECRETION PRODUCT

Examiner: R. Schwadron

Group Art Unit: 1644

DECLARATION OF MARIO ASSENMACHER PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Mario Assenmacher, declare as follows:

1. I am a co-author of a manuscript, which discusses the claimed technology, with one of the inventors of the above-referenced patent application, and as such am familiar with the claimed subject matter. I have read the Office Action dated December 9, 1997 and understand that the Examiner would like to see further support for the fact that cells can be separated according to the methods of the present invention without using a high viscosity or gel forming medium. The following experiments were performed at my direction and that of inventor A. Radbruch and show selection of IL-2-secreting cells by labeling these cells on the basis of

relocating secreted IL-2 to the cell surface without using viscosity enhancing agents. A copy of my Curriculum Vitae is attached as Exhibit 1.

2. Selection of IL-2-secreting T cells

In vivo and *in vitro,* the primary T cell response to staphylococcal enterotoxin B (SEB) is characterized by a T helper cell type 1 (Th1) cytokine pattern, with prominent expression of interleukin-2 (IL-2), tumor necrosis factor-α and interferon-γ (IFN-γ) versus low expression of IL-10, resembling infections with a variety of intracellular pathogens. It has previously been shown by intracellular immunofluorescence that upon primary stimulation of murine splenic Th cells with SEB *in vitro,* IL-2, IFN-γ and IL-10 are expressed with fast, intermediate and slow kinetics, respectively, in the population of activated Th cells. Similar kinetics of cytokine expression, *i.e.* early IL-2, and later IFN-γ and IL-10, have been found upon exposure to SEB *in vivo*. To isolate IL-2-secreting cells from day 1 of primary SEB stimulation for analysis of their cytokine expression upon continued SEB culture, recently developed technologies were employed for the separation of live cells according to the cytokines they express. IL-2-secreting cells were separated after labeling by cellular affinity matrix technology.

Pre-sorting and antigen stimulation of spleen cells

Specific pathogen-free BALB/cJ mice, 8-12 weeks old, were obtained from Bomholtgard Breeding and Research Centre LTD (Ry, Denmark). Mice were killed by cervical dislocation and spleen cells (SC) were prepared. In most experiments, $CD8^+$ T cells were depleted by high-gradient magnetic cell separation with MACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). In one experiment, $CD4^+$ $CD62L^+$ cells were positively selected from SC by MACS MultiSort (Miltenyi Biotec). SC depleted of T cells to less than 0.5% were used as antigen presenting cells (APC). T cells were depleted with anti-CD4-, anti-CD8- and anti-CD5-microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Cells were cultured in complete RPMI 1640 (Gibco BRL, Grand Island, NY) containing 100U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine, 10 μM 2-ME and 5% FCS (FAA, Linz, Austria) at 1-2 x 10^6 cells/ml. SEB (Sigma, St. Louis, MO) was added at 2 μg/ml.

Labeling of cells based on secretion of IL-2

IL-2-secreting cells were detected using the modified cell-surface affinity matrix technique. About 10^8 cells were biotinylated in 1 ml of sulfosuccinimidyl 6-(biotinamido)

hexanoate (1 mg/ml; Pierce) in phosphate-buffered saline (PBS), at pH 8.4. After 10 minutes at room temperature and 5 minutes at 37°C, the cells were washed twice in PBS with 0.5% bovine serum albumin (BSA), transferring the cells to a new tube once. The cells were incubated at 4°C for 5 minutes with avidin-conjugated JES6-1A12 (25 μg/ml) in PBS/BSA with 0.01% sodium azide (NaN_3), to label the cell surface with an affinity matrix for murine IL-2. JES6-1A12 is a rat monoclonal antibody specific for mouse IL-2. The cells were then incubated for secretion in complete RPMI/FCS/2-ME at a concentration of 10^6 cells/ml. The requirement for embedding the cells in media of high viscosity during the secretion period was overcome by optimizing incubation conditions with respect to cell density and incubation time. After 60 minutes, the cells were spun down, and IL-2 bound to the cell-surface affinity matrix (JES6-1Al2) was labeled with digoxigenin (DIG)-conjugated JES6-5H4 (5 μg/ml) in PBS/BSA/NaN_3, for 10 minutes at 4°C. JES6-5H4 is a rat monoclonal antibody specific for mouse IL-2. Cells were then washed twice in PBS/BSA/NaN_3 and stained with sheep anti-DIG Fab-conjugated magnetofluorescent liposomes in PBS/BSA/NaN_3 for 30 minutes at 4°C, with gentle agitation. Cells were washed twice, resuspended in PBS/BSA/NaN_3 and analyzed by flow cytometry.

Analysis of labeled cells

After 1 day of SEB stimulation, IL-2-secreting cells were labeled using the cellular affinity matrix technology described above, and analyzed before being separated alive by MACS. The results of the analysis are shown in Figure 1.

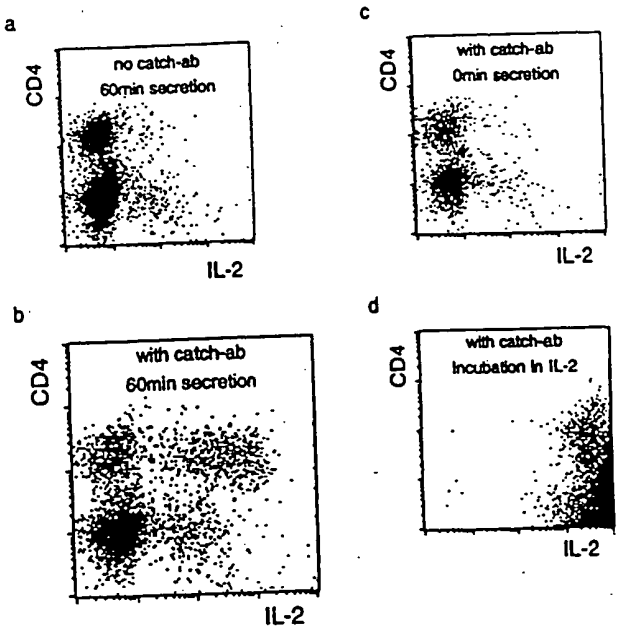

Figure 1. Detection of IL-2-secreting Th cells using the cell-surface affinity matrix technology. CD8-depleted spleen cells (SC) were stimulated with SEB for 18 hours and then biotinylated. The biotinylated cells were labeled with an avidin-conjugated anti-IL-2 MAb (catch-Ab) and allowed to secrete IL-2 for 60 minutes at 37°C. Then, bound IL-2 was detected by a digoxigenized second anti-IL-2 mAb (detection-Ab) and DIG-specific magnetofluorescent liposomes. Dot plots show surface staining of IL-2 versus CD4 on large activated lymphocytes (blasts), gated according to forward and side scatter, with exclusion of dead cells by gating against PI staining (b). As negative controls, affinity matrix-, *i.e.* catch-Ab-coated cells without incubation at 37°C (c) and cells without catch-Ab labeling but 60 minutes time for secretion at 37°C (a) were stained for secreted IL-2 versus CD4. As high control, cells with catch-Ab labeling incubated in rIL-2 for 10 minutes (d) were stained for IL-2 versus CD4.

Secreted IL-2 was detected mainly on $CD4^+$ blasts (Fig. 1b). About 40% of $CD4^+$ blasts clearly stained for secreted IL-2 (Fig. 1b). Only 3% of small $CD4^+$ lymphocytes were stained (data not shown) and $CD4^-$ cells displayed only less bright background staining (Fig. 1b). This restricted staining pattern with respect to cell type and activation status confirms specificity of the staining for secreted IL-2. Furthermore, intracellular counterstaining showed that surface staining of the secreted cytokine corresponds entirely to the intracellular staining of this product (data not shown). Negative controls, *i.e.* cells without an affinity matrix (Fig. 1a) and cells with an affinity matrix but no time for secretion (Fig. 1c), showed only marginal background staining (preferentially in the $CD4^-$ fraction) (Fig. 1a, c). A high control of cells with an affinity matrix and incubated in recombinant IL-2, was homogeneously and brightly stained (Fig. 1d).

Enrichment of IL-2-secreting cells

IL-2-secreting cells were enriched by MACS on Vs^+ columns. IL-2-secreting cells were enriched from 8.8% of large lymphocytes before to 95% after MACS (Fig. 2) and from 2% of all cells to 74%. Since IL-2-secreting cells were mainly large blast-like cells, the frequency of blasts among all cells was increased 2.5-fold from about 20% to about 50% by selection of IL-2-secreting cells (data not shown). In the negative population, 5.2% of blasts and 1% of all lymphocytes were stained for secreted IL-2 (data not shown).

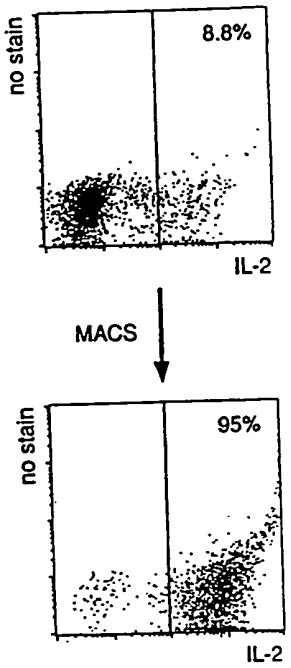

Figure 2. Enrichment of IL-2-secreting cells. After 18 hours of stimulation with SEB, CD8-depleted SC were stained for secreted IL-2 using magnetofluorescent liposomes, as described in Fig. 1. Stained cells were passed over a magnetized Vs^+ MACS column. After washing, positive cells were eluted from the demagnetized column. Dot plots show staining of secreted IL-2, before and after MACS, gated as described in Fig. 1.

IL-2 secretion by enriched cells

Unseparated cells, the fraction of cells selected for secreted IL-2, and the negative cell fraction were cultured further. One day later, *i.e.* 2 days after onset of SEB stimulation, the concentration of IL-2 in the culture supernatants was determined. Concentrations of IL-2 in culture supernatants were determined by sandwich ELISA, using the mAb JES6-5H4 as coating Ab and the biotinylated mAb JES6-1A12 as developing Ab. The results are shown in Figure 3.

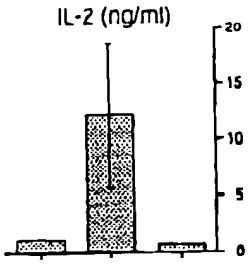

Figure 3. Enriched cells secrete IL-2. After 1 day of stimulation with SEB, IL-2-secreting cells were isolated alive as shown in Figs. 1 and 2. Unseparated (0), IL-2-positive cells (+) and IL-2-negative cells (-) were cultured further and analyzed for subsequent cytokine production by ELISA. Cells from the IL-2 separation were analyzed for the production of IL-2 on day 2 following enrichment.

The concentration of IL-2 in the supernatant of enriched IL-2-positive cells was 11-fold higher than in the culture supernatant of unseparated cells and 20-fold higher than in the supernatant of IL-2-negative cells (Figure 3), confirming the successful enrichment of IL-2-secreting cells.

Taken together, these results demonstrate that detection and enrichment of IL-2-secreting cells can be achieved using separation techniques based on capture of IL-2 to the cell surface of IL-2-secreting cells without the addition of high viscosity media.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

4.6.98
Date

Mario W. Assenmacher